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TITLE: Low-Voltage Activated Calcium Channels - Their Role in HER2-Driven Breast Cancer and Potential as a New Therapeutic Target

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14. ABSTRACT

Breast cancer is a devastating disease that affects both women and men, and despite many advancements it is still often deadly and remains incurable when metastatic. Efficient novel treatments, or enhancements to current ones, are desperately needed to improve breast cancer therapy and to extend the lives of patients. Therefore, the overall goal of this proposal is to develop a tumor-specific, safe and effective therapy for breast cancer. We concentrate on HER2-overexpressing tumors, which are diagnosed in one in four patients. Currently HER2 targeted therapies achieve highest response rates when combined with chemotherapy, but chemotherapy causes undesirable side effects due to off-target effects on normal tissue, which diminishes quality of life for the patient. One way to address this problem is to use a drug that either attacks only tumor cells or enhances the response of tumors (but not normal tissues) to current therapies. During the first year of this project we demonstrated that so called low voltage activated (LVA) calcium channels are aberrantly expressed in HER2-positive breast cancer cell lines, in PyMT mouse tumors-derived cell lines, and contribute to worsen prognosis in human breast cancer patients (analysis of publicly available data). We showed that inhibition of LVA channel with clinically approved agent mibefradil reduces oncogenic signaling and enhances anticancer activity of standard therapeutic agents. Similar, although not as pronounced, effects were observed in cell lines with down-regulated expression of LVA channel isoform CACNA1G. In summary, the results from the 1st year of this project provide foundation for future more extensive molecular studies *in vitro*, and efficacy studies *in vivo*.

15. SUBJECT TERMS

breast cancer, calcium channel, HER2-positive, chemotherapy, mibefradil

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Annual Report for the Project: **W81XWH-15-1-0308**
October 2016

Project: **Low-Voltage Activated Calcium Channels - Their Role in HER2-Driven Breast Cancer and Potential as a New Therapeutic Target**

PI: Jaroslaw Dziegielewski, PhD
University of Virginia

1. Introduction

Breast cancer is a devastating disease that affects both women and men, and despite many advancements it is still often deadly and remains incurable when metastatic. Efficient novel treatments, or enhancements to current ones, are desperately needed to improve breast cancer therapy and to extend the lives of patients. Therefore, the overall goal of this proposal is to develop a tumor-specific, safe and effective therapy for breast cancer. We concentrate on HER2-overexpressing tumors, which are diagnosed in one in four patients. Currently HER2 targeted therapies achieve highest response rates when combined with chemotherapy, but chemotherapy causes undesirable side effects due to off-target effects on normal tissue, which diminishes quality of life for the patient. One way to address this problem is to use a drug that either attacks only tumor cells or enhances the response of tumors (but not normal tissues) to current therapies. Our preliminary research shows that so called low voltage activated (LVA) calcium channels are aberrantly expressed in breast cancer cells, most notably in HER2-positive tumors. Here, we investigate how expression of LVA calcium channels may promote cancer cell growth and progression and confer resistance to therapy. We also determine whether LVA channel inhibitors, mibefradil and similar drugs, can be employed to treat an advanced HER2-positive breast cancer. Our proposed research is expected to likely reveal an important mechanism(s) that support the progression of this aggressive HER2+ breast cancer. The successful outcome of this project will advance the rational approach to develop a new generation of anticancer agents for the treatment of patients suffering from advanced breast tumors.

2. Keywords

breast cancer, HER2, calcium channel, chemotherapy, mibefradil
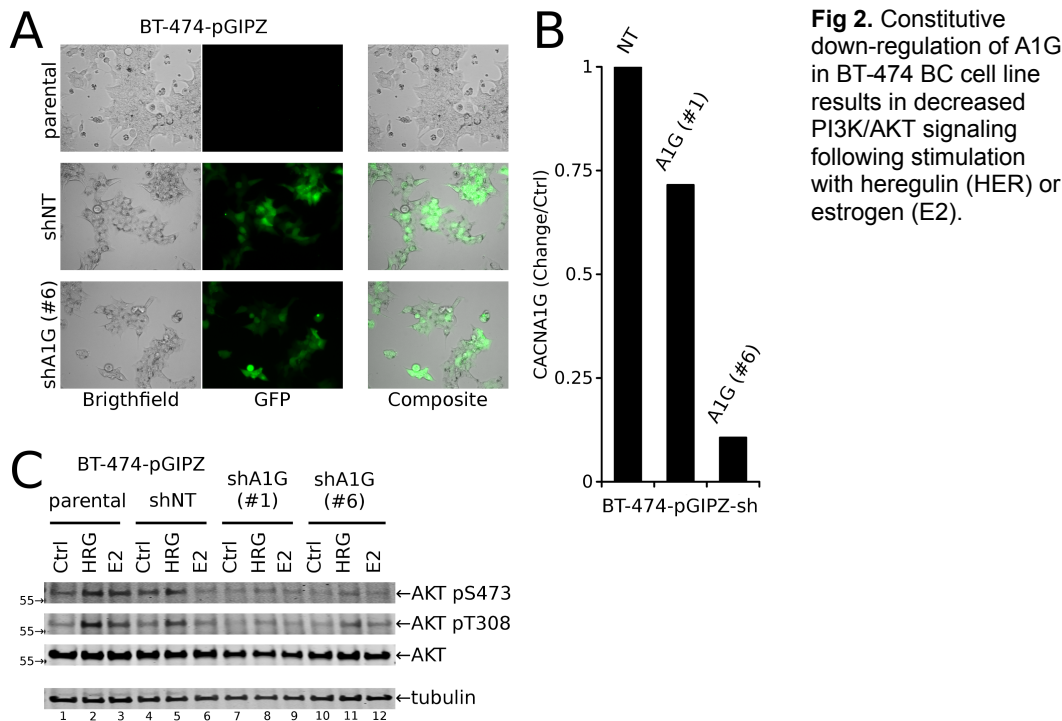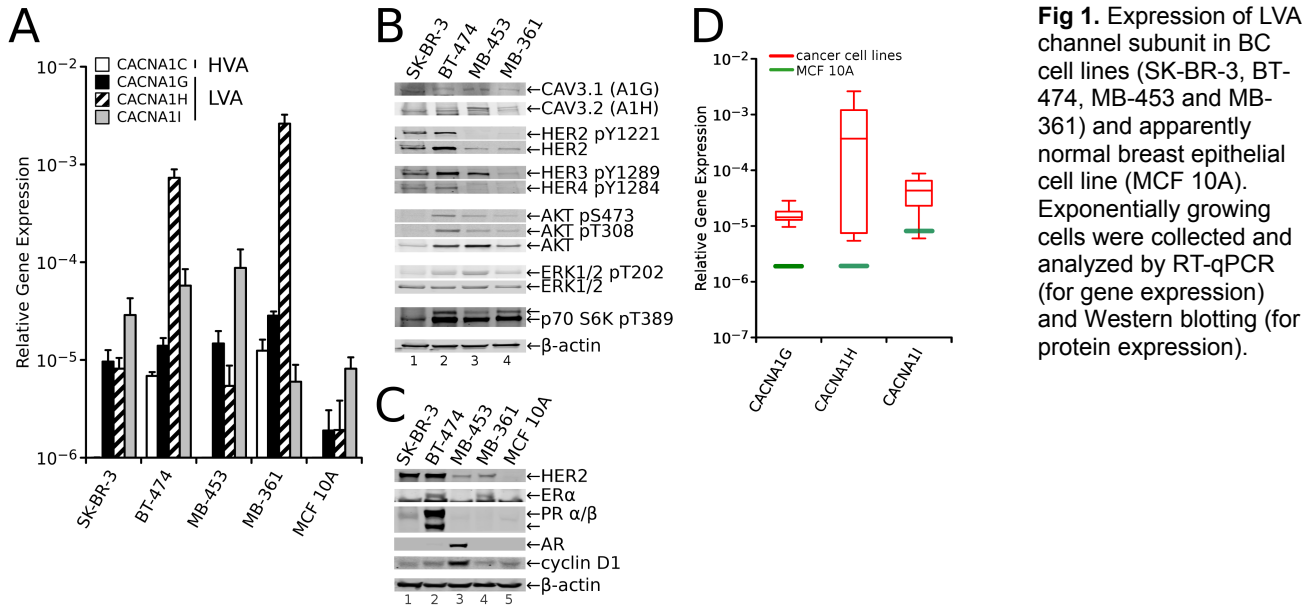
3. Accomplishments

What were the major goals of the project?

This is the first year report covering the initial phase of the project, during which we have concentrated on initial sub-tasks of Major Tasks 1 to 5, as delineated in the approved Statement of Work. The major goal for the first year of the project was to test the importance of LVA channels in HER2-positive breast cancer (BC), using cell lines, animal models and data derived from analysis of human samples.
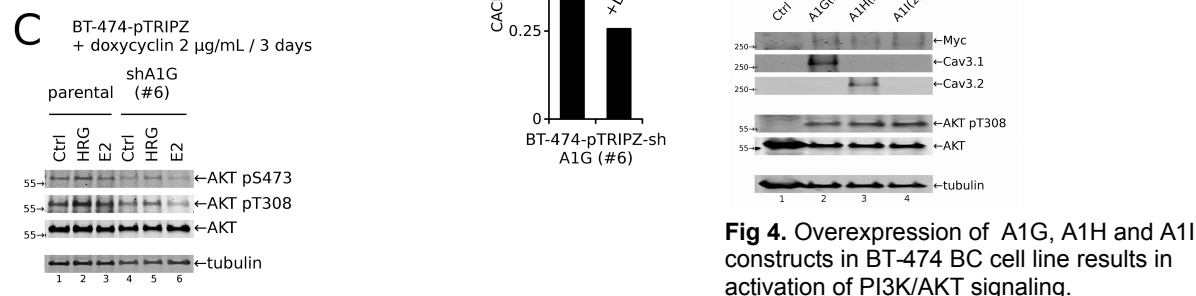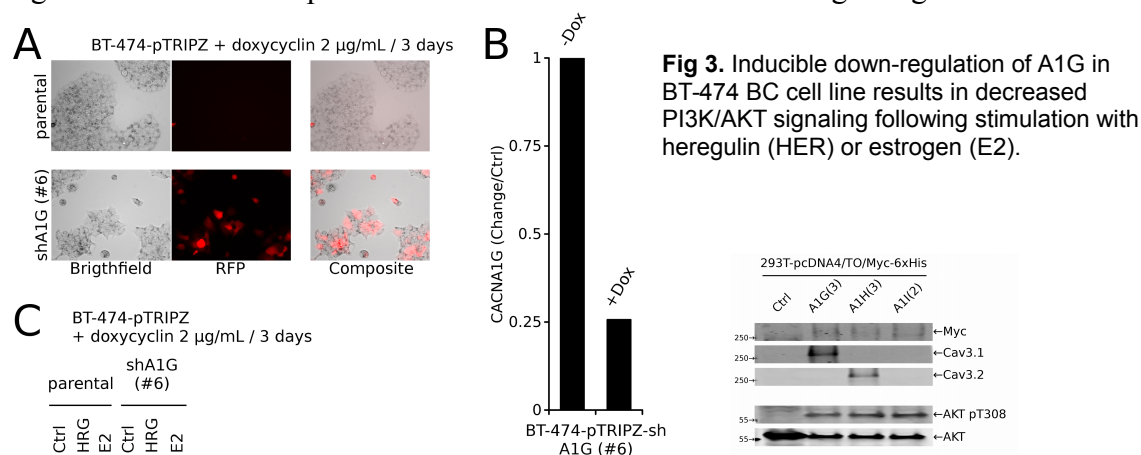
What was accomplished under these goals?

3.1. Major Task 1: Characterize expression of LVA channels in selected BC cell lines differing in HER2 expression and sensitivity to anti-HER2 therapy.
Subtasks 1.1, months: 1-3. Completion: 100%
Subtask 1.2, months: 3-9. Completion: 50%

As shown in **Fig 1,** four tested BC cell lines express isoforms of A1 subunit (A1G, A1H and A1I) of LVA calcium channel. Importantly, there is a significant difference in channel expression between cancer cell lines, and apparently normal breast epithelial cell line MCF 10A (**Fig 1D**). (Subtask 1.1)

Fig 1. Expression of LVA channel subunit in BC cell lines (SK-BR-3, BT-474, MB-453 and MB-361) and apparently normal breast epithelial cell line (MCF 10A). Exponentially growing cells were collected and analyzed by RT-qPCR (for gene expression) and Western blotting (for protein expression).

Fig 2. Constitutive down-regulation of A1G in BT-474 BC cell line results in decreased PI3K/AKT signaling following stimulation with heregulin (HER) or estrogen (E2).

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Next, we down-regulated (knock-down) LVA channel subunit A1G in breast cancer cells, using pGIPZ (constitutive expression, with GFP marker) and pTRIPZ (doxocylin-inducible expression, with RFP marker) shRNA lentiviral systems (Dharmacon/GE Life). As demonstrated in **Fig 2.** and **Fig 3.** expression of shRNA targeted against A1G isoform results in down-regulation of mRNA expression and attenuation of PI3K/AKT signaling.

Fig 3. Inducible down-regulation of A1G in BT-474 BC cell line results in decreased PI3K/AKT signaling following stimulation with heregulin (HER) or estrogen (E2).

Fig 4. Overexpression of A1G, A1H and A1I constructs in BT-474 BC cell line results in activation of PI3K/AKT signaling.

We have created plasmids (based on pcDNA4/TO/myc-His, ThermoFisher) harboring cDNA for A1G, A1H and A1I isoforms, tagged with myc-tag and His-tag. Our attempts at overexpressing LVA constructs in BC cells were unsuccessful so far, therefore first we tested such overexpression in 293T cells (human embryonic kidney cells, a well established standard molecular biology / biochemistry tool). As shown in **Fig 4.** overexpression of all three isoforms of A1 subunit (A1G, A1H and A1H) increases PI3K/AKT signaling in 293T cells.

3.2. Major Task 2: Test the clinically available LVA channels inhibitor mibefradil on BC cells in combination with anti-HER2 treatments and/or chemotherapy.
Subtask 2.1, months: 10-12. Completion: 100%
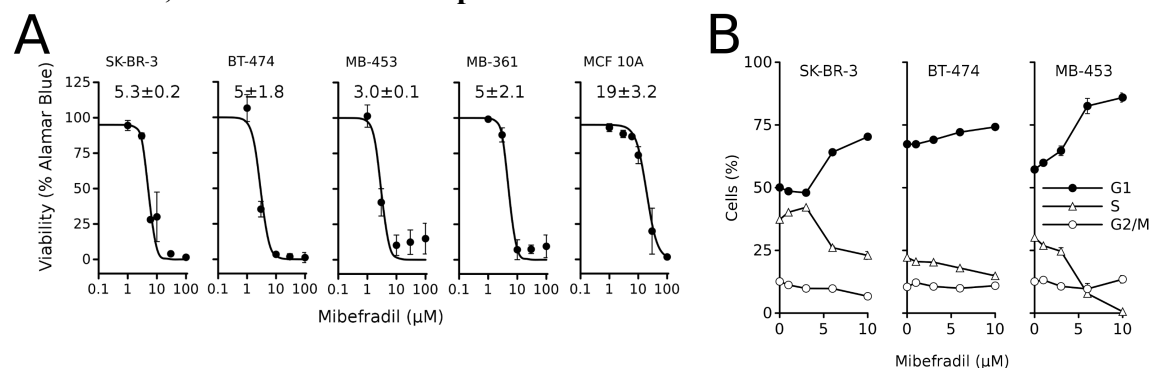**Subtask 2.2, months: 12-16. Completion: 50%**

Fig 5. Mibefradil, a LAV inhibitor, reduces cancer cells viability and arrest their cell cycle progression in G1 phase. **A)** Cells were treated with increasing concentrations of mibefradil for 72 h and the viability assessed by Alamar Blue assay. **B)** Cells were treated with increasing concentrations of mibefradil for 24 h and cell cycle progression assessed by BrdU incorporation and PI staining.

We have tested mibefradil effects on cells' viability and HER2-related oncogenic signaling in several BC HER2-positive cell lines *in vitro*. As shown in **Fig 5.** all BC cell lines are sensitive to the drug, with EC$_{50}$ in the range of 3 to 5 µM. Importantly, the apparently normal breast epithelial cell line MCF 10A is at least four times more resistant to mibefradil (EC$_{50}$ = 19 µM). Moreover, treatment with the drug reduces constitutive activation of PI3K/AKT/mTOR pathway(s) and induces cell death (apoptosis) in cancer cells (**Fig 6.**). Those effects are not observed in apparently normal MCF 10A cells.
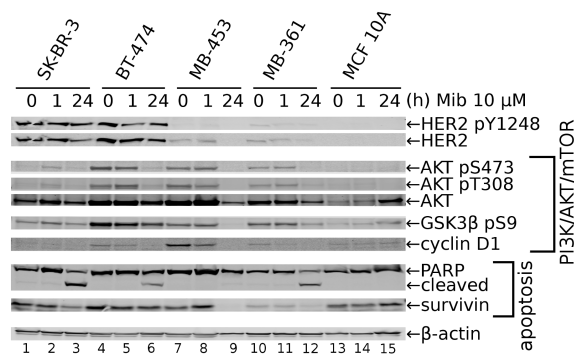

Fig 6. Mibefradil decreases constitutive activation of PI3K/AKT/mTOR pathway(s) and induces apoptosis in cancer cells. Cells were treated with 10 µM drug for 1 or 24 h, then assessed for the expression of protein markers by Western blot.

We have assessed the effects of LVA channel inhibition in combination with current chemotherapeutics used in HER2-positive cancer. As demonstrated in **Fig 7.** combination treatment enhances pro-apoptotic effects of Lapatinib (Lnib, HER2 kinase inhibitor) and Paclitaxel (Ptx, anti-microtubule agent).
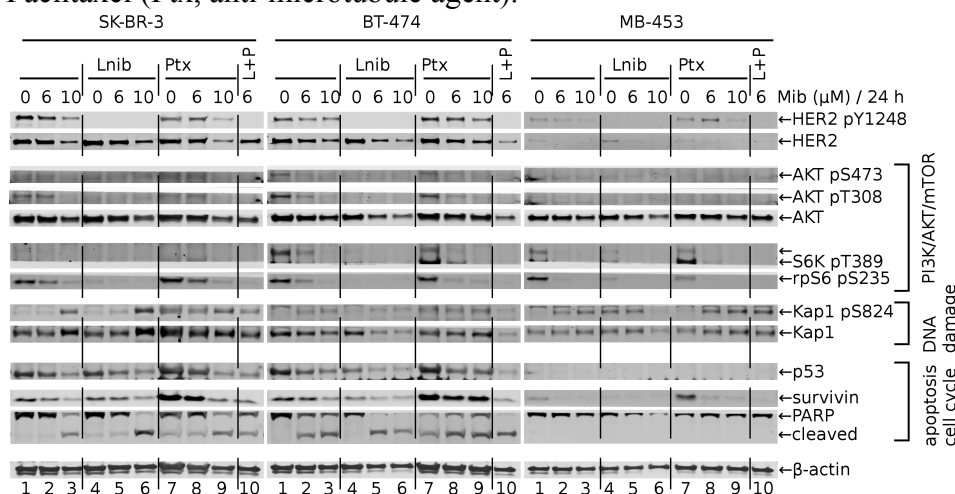

Fig 7. Mibefradil inhibits PI3K/AKT/mTOR pathway(s) and enhances cell death induced by lapatinib (Lnib) or pacitaxel (Ptx) in HER2-positive cancer cells. Cells were treated with 0, 6 or 10 µM drug for 24 h in the presence of 1 µM Lnib or 100 nM Ptx (or both drugs , L+P), then assessed for the expression of protein markers by Western blot.

3.3. Major Task 3: Elucidate the molecular mechanism(s) of action of LVA channels inhibitors. **Subtask 3.1, months: 6-24. Completion: 30%**
Subtask 3.2, months: 12-24. Completion: 30%

Since HER2 receptor can be activated by heterodimerization with other members of HER family, activated through binding with appropriate ligand (e.g. EGFR / EGF, HER3 / heregulin), or through cross-talk with other receptor(s) (e.g. estrogen receptor and estrogen, InsR and insulin), we have studied the effects of LVA channel inhibition on trans-activation of HER2. Serum-starved BT-474 cells (HER2/ER-positive) were cultured for 24 h in the presence of serum (lanes 1-2), EGF, insulin (Ins) or estrogen (E2). As demonstrated in **Fig 8.** in all cases these

stimulations resulted in increased phosphorylation of HER2 and activation of downstream pathways (PI3K/AKT/mTOR, cell cycle). However, co-treatment with mibefradil, even at relatively low concentration of 6 μM, significantly reduced both activation of HER2 and its downstream effectors.
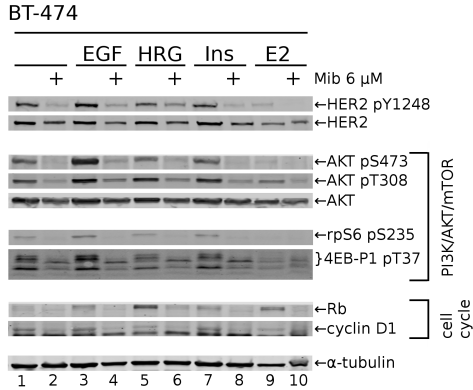

Fig 8. Mibefradil inhibits activation of HER2 and its downstream effectors in BT-474 cancer cells stimulated with EGF, hereguli, insulin or estogen.

3.4. Major Task 4: Evaluate the expression of LVA channels in samples from HER2+ breast cancer patients. **Subtask 4.1, months: 1-3. Completion: 0%**
Subtask 4.2, months: 4-36. Completion: 0%

Due to technical problems with validation of anti-LVA channel antibodies for immunohistochemistry in human samples, we were unable to start this part of the project (please see the "Problems" section). In preparation for this study, we have located all HER2-positive patient samples at the UVA Pathology (CDR, Clinical Data Repository, anonymized samples) and are ready to analyze them pending IRB approval and antibody validation.

Because we were unable to access human samples from local repository, we have analyzed publicly available data from Breast Mark repository, Molecular Therapeutics for Cancer, Ireland (http://glados.ucd.ie/BreastMark/). As shown in **Fig 9.** co-expression of HER2 and CACNA1G isoform reduces Disease-Free Survival (**Fig 9A**) and increases Hazard Ratio (**Fig 9B**) in patients suffering from any molecular type of breast cancer (PAM50).
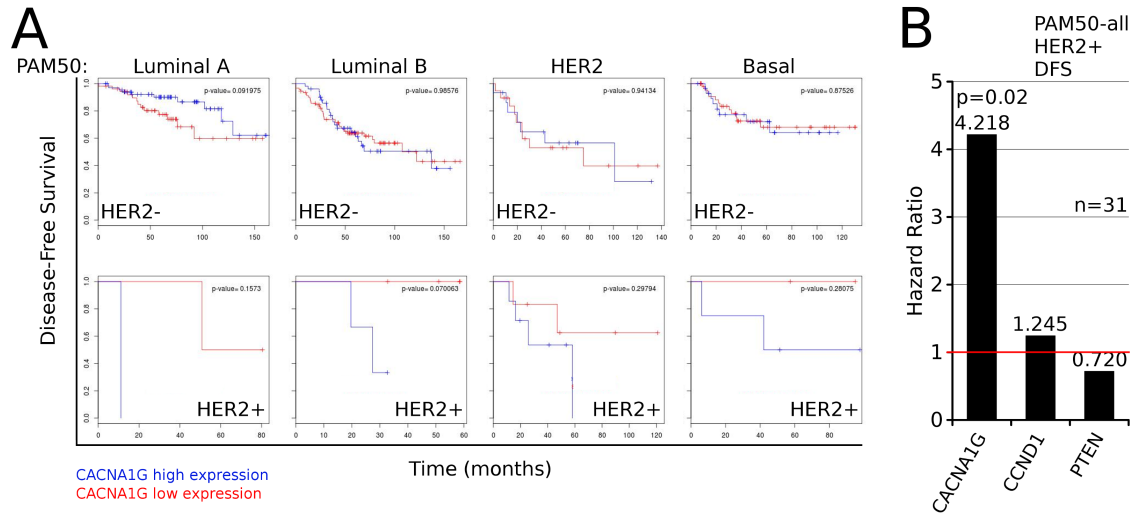

Fig 9. High expression of CACNA1G in HER2-positive cancer reduces Disease-Free Survival and increases Hazard Ratio in breast cancer patients. Analysis using publicly available data from Breast Mark (http://glados.ucd.ie/BreastMark/).

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3.5. Major Task 5: Characterize the role of LVA channels in animal models of HER2+ BC. **Subtask 5.1, months: 1-3. Completion: 100%**
Subtask 5.2, months: 4-24. Completion: 10%

Due to similar problems with antibodies validation, we were unable to initiate on time this part of project. However, the subtask 5.1 is now completed (Animal Research Protocol prepared and approved by UVA IACUC and DOD USAMRMC), and the PyMT mice colony has been started. In the meantime we have assessed the expression of LVA channels in cell lines derived from PyMT tumors (**Fig 10A**), as well as the cells sensitivity to LVA inhibition (**Fig 10B**). As shown in **Fig 10** cell line Py230 (primary from mice tumors, luminal type) express both HER2 and CACNA1G. The Py117 (differentiated into basal type) express lower level of CACNA1G, while in Py15-4 (differentiated into claudin-low) the expression of CACNA1G is undetectable using our method.
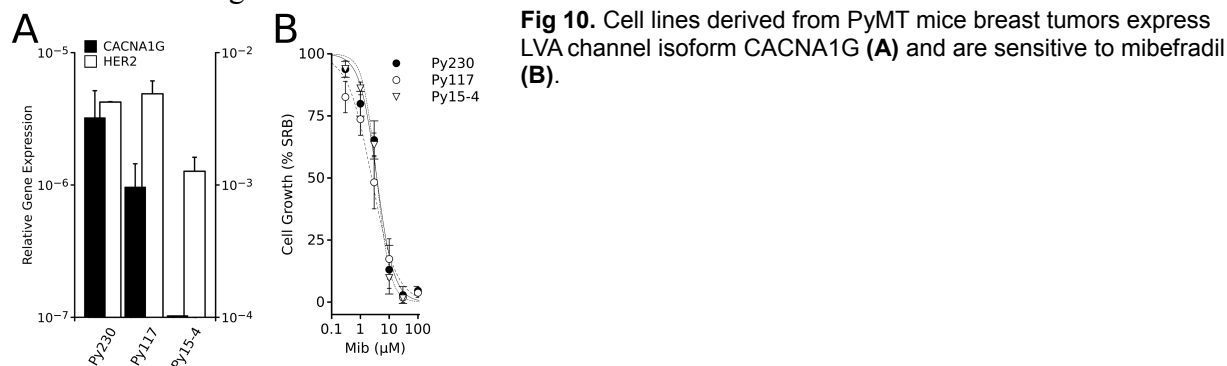

Fig 10. Cell lines derived from PyMT mice breast tumors express LVA channel isoform CACNA1G **(A)** and are sensitive to mibefradil **(B)**.

Finally, we have analyzed several PyMT mice breast tumor tissue samples donated by our colleagues, demonstrating over-stimulation of AKT activity (**Fig 11**). Based on our in vitro results, inhibition of AKT activation could be used as an activity marker of anti-LVA channel therapy.
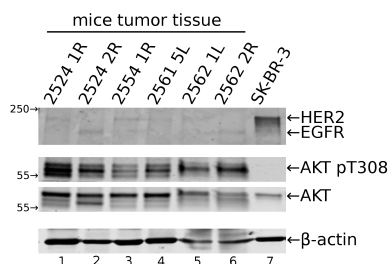

Fig 11. Tissue samples from PyMT mice tumors display over-activation of AKT.

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?
Because the technical problems with validation of anti-LVA antibodies for IHC in both mice and human samples were the major obstacles with accomplishing all subtasks/milestones scheduled for the 1st year of this project, we have initiated custom production of anti-Cav3.1 (CACNA1G) antibody (GenScript). The initial testings of immune serum are very promising,

and we are awaiting final delivery of purified antibody. Moreover, we have developed cell lines with either over-expression (Myc/His-tag) or down-regulation (GFP/RFP-markers) of LVA expression, which can be used as positive and negative controls, respectively, for the antibody validation. We are also preparing IRB application, to be submitted for UVA and DOD approval before the end of 2016.

For the Major Task 5 (animal studies) we are planing to finish Subtask 3.2, and start Subtask 3.1 as soon as possible.

For the *in vitro* studies we will continue work as delineated in the SOW.

Finally, we plan to participate in 2017 AACR Annual Meeting and San Antonio Breast Cancer meeting to disseminate our discoveries to research and patients communities.

4. Impact

We believe that our findings about the role of LVA channels in HER2-positive breast cancer are important for developing new and better therapies for this deadly tumor. Because our findings were not published or disseminated in any way outside of internal seminars and conferences, they have not yet had the desired impact on the medical research community.

5. Changes/Problems

Actual or anticipated problems or delays and actions or plans to resolve them.

As described above, problems with antibody validation significantly slowed down the work on Aim 3 – studies in mice model and analysis of patient samples. We are rectifying this problem by producing custom antibody (commercial service by GenScript, Inc.) against specific and validated antigenic peptide sequence.

Significant changes in use or care of human subjects.

We have planed to develop IRB and start analysis of patients samples during 1st year of this project. Due to problems with antibodies this part of the project is postponed to 2nd year.

Significant changes in use or care of vertebrate animals.

We have planed to initiate animal experiments in 4th months of this project. Due to technical problems these experiments were delayed for approximately 7 months. Currently, we have Animal Protocol approved by both IACUC and DOD, and the first animal experiment has been started.

6. Products

Nothing to report.

7. Participants & Other Collaborating Organizations

Name:	Jaroslaw Dziegielewski, Ph.D.
Project Role:	PI
Person-months worked:	6
Contribution to Project:	Dr. Dziegielewski designed all experiments, performed some of in vitro experiment, participated in analysis of the results, and prepared the Annual Report

Name:	Amy H. Bouton, Ph.D.
Project Role:	Collaborator
Person-months worked:	<1
Contribution to Project:	Dr. Bouton participated in the design and analysis of all experiments. She also provided mice tumor samples and cell lines, as well as reagents and expertise to analyze animal samples.

Name:	Patrick M. Dillon, M.D.
Project Role:	Collaborator
Person-months worked:	<1
Contribution to Project:	Dr. Dillon participated in the design and analysis of all experiments. He also provided the expertise to analyze human studies.

Name:	Barbara Dziegielewska, Ph.D.
Project Role:	Key Personnel, Research Scientist
Person-months worked:	6
Contribution to Project:	Dr. Dziegielewska participated in the design of all experiments, performed some of in vitro experiment, and participated in analysis of the results. She is also responsible for mice breeding colony.

Name:	William T. Warnock
Project Role:	Key Personnel, Research Assistant
Person-months worked:	6
Contribution to Project:	Mr. Warnock participated in the design of all experiments, performed some of in vitro experiment, and participated in analysis of the results.

8. Special Reporting Requirements

Nothing to report.

9. Appendices

Nothing to report.